PATENT

BI FUSION PROTEINS

MULTIMERIC FORMS OF TNF SUPERFAMILY LIGANDS

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a method form preparing soluble multimeric proteins consisting of more than three iterations of the same bioactive molecule using recombinant DNA technology.

The present invention particularly concerns a new method of producing multimeric fusion proteins involving the TNF superfamily (TNFSF) members as a fusion proteins with SPD, and more specifically, CD40L-SPD fusion proteins and useful modifications thereof.

Description of Related Art

Numerous proteins can be made using modern molecular biology techniques and used in diagnostic and therapeutic applications. Using recombinant DNA techniques, the DNA encoding a single amino acid chain is constructed and then introduced into a cell which manufactures the final protein. Some cells, especially bacteria like *E. coli,* lack the ability to properly fold the amino acid chains into the proper quaternary structure and they often fail to apply the necessary modifications (e.g., glycosylation and disulfide bond formation) that are needed for the protein to be bioactive and resistant to degradation *in vivo*.

While most of these challenges can be met by expressing the amino acid chain in eukaryotic cells like yeast or mammalian cells *in vitro*, it is not always straightforward to express proteins that consist of two or more amino acid chains. In general, for multichain proteins, the single amino acid chains must associate together in some way either within the producer cell or subsequently after the monomers are secreted from the producer cell. For artificially constructed molecules, the introduction into a single amino acid chain of an amino acid sequence which causes this chain-to-chain association can be an important step in producing multichain proteins.

One of the most widely used methods of causing two amino acid chains to associate is to conjoin, at the DNA coding level, segments from the protein of interest and a segment from a spontaneously dimerizing protein. The best example is to conjoin or fuse a protein with the Fc portion of immunoglobulin, creating a dimeric Fc fusion protein (Fanslow *et al.*, *J. Immunol.* 136:4099, 1986). A protein of this type can be formed from the extracellular domain of a tumor necrosis factor receptor fused to Fc (termed etanercept and marketed as ENBREL®), which is effective in the treatment of rheumatoid arthritis. A second example is the construction of a fusion protein between the dimerizing extracellular portion of CD8 with the extracellular portion of CD40L (Hollenbaugh *et al.*, *EMBO J.* 11:4313, 1992). Here, the dimerizing CD8 portion of the fusion protein helps to maintain the CD40L portion in the trimeric form needed for its bioactivity. A more recent example is the addition of an isoleucine zipper motif to CD40L, which permits the production of trimeric soluble CD40L molecules (Morris *et al.*, *J. Biol. Chem.* 274:418, 1999).

The TNF superfamily (TNFSF) consists of an expanding number of proteins

(see Table I) which are crucial for the development and functioning of the immune, hematological, and skeletal systems. TNFSF proteins are ligands for a corresponding set of receptors of the TNF receptor superfamily (TNFRSF). All TNFSF members are expressed as Type II membrane proteins, with the exception of lymphotoxin-alpha which is produced as a secreted protein. However, soluble forms of several TNFSF proteins can be released from the cell surface by proteolytic cleavage, usually by specific metalloproteinases.

The production of soluble forms of TNFSF proteins has been an important step in the study of these proteins. Soluble TNFSF ligands can be used to study the activities of these proteins in vitro without the complexities in interpretation that result when cells or cellular membranes expressing TNFSF proteins are added. In addition, soluble forms of several TNFSF proteins have potential as therapeutic agents for human diseases. In particular, TNF-? has been extensively studied for the treatment of cancer and soluble CD40L is currently undergoing clinical trials to assess its antitumor effects.

To produce soluble forms of TNFSF proteins, either the membrane protein is expressed in a cell line possessing a protease capable of separating the TNFSF extracellular domain from the transmembrane domain or a truncated form of the TNFSF protein is produced which consists solely of the extracellular domain plus a signal sequence. In either case, certain soluble forms of TNFSF proteins are unstable in solution as simple homotrimers composed solely of the extracellular domain. For example, naturally solubilized TNF-? is labile under physiological conditions

[Schuchmann, 1995 #129]. To solve this stability problem, chimeric proteins have been constructed according to one of four different design principles: (1) The extracellular portion of the TNFSF protein has been expressed fused to the dimeric portion of the immunoglobulin Fc fragment US Patent 5,155,027, Oct. 13, 1992, issued to, Andrzej Z. Sledziewski, et al. In the case of CD40L and OX40L, this yields a soluble molecule which is significantly less active than the native membrane form of this protein. (2) The extracellular portion of the TNFSF protein has been expressed with an antigenic tag (usually the FLAG motif) fused to its N-terminus [Mariani, 1996]. The addition of an antibody to the tag (e.g., anti-FLAG antibody) aggregates these proteins into a multimeric form. Crosslinking enhances activity on B cells. (3) The extracellular portion of the TNFSF protein has been expressed fused to the spontaneously dimerizing extracellular portion of the CD8 molecule [Hollenbaugh, 1992]. In the case of CD40L, this creates a hexameric molecule [Pullen, 1999] which is likely formed by two CD40L trimers attached to three CD8 dimeric stalks. Despite this, the addition of an anti-CD8 antibody to crosslink the CD40L-CD8 fusion protein yields a further enhancement of CD40L activity on B cells. (4) The extracellular portion of the TNFSF protein has been expressed fused to a trimerizing isoleucine zipper which maintains the overall trimeric structure of the protein [US Patent5,716,805, Feb. 10, 1998, issued to Subashini Srinivasan et al. This soluble CD40L trimer or 'sCD40LT' is the form of that protein now being clinically tested in humans for its anti-tumor effects.

Compounding the difficulties in producing stable forms of soluble TNFSF proteins are compromises in bioactivity. As exemplified by FasL, TNF, and CD40L, many of the

soluble forms of these proteins lack the full range of stimulatory activities displayed by the membrane forms of these molecules. For FasL, several groups have reported that naturally produced soluble FasL (generated by proteolytic cleavage from the membrane form) has a spectrum of activities that is distinctly different from the membrane form. Soluble FasL induces apoptosis in activated CD4+ T cells but not fresh, resting CD4+ T cells. In contrast, both types of CD4+ T cells are killed by membrane FasL or a recombinant soluble form of FasL (WX1) that spontaneously aggregates into oligomers larger than a decamer. For TNF, T cell activation through stimulation of TNFR II, the 80 kDa receptor for TNF, is much greater with membrane TNF than soluble TNF. However, if soluble TNF is produced as a tagged protein and crosslinked with an antibody against the tag, then it completely mimics the activities of membrane TNF [Schneider, 1998]. Finally, for CD40L, the stimulatory effects of a soluble form of this TNFSF protein are enhanced by crosslinking [Kehry, 1994] and yields an activity similar to membrane CD40L. For example, soluble CD40L-CD8 fusion protein requires crosslinking with a antibody to CD8 in order to drive resting B cells to proliferate to a degree similar to membrane-bound CD40L.). Even more strikingly, although membrane-bound CD40L expressed on baculovirus-transduced SF9 insect cells is a strong B cell stimulus, small vesicles (10 - 1,500 nm) prepared from the membranes of these cells are less stimulatory. However, ultracentrifugation of these vesicles creates aggregates which have the full activity of the original membrane CD40L protein. This indicates that B cells are more highly stimulated by a large surface of CD40L than they are by a smaller surface expressing this membrane ligand.

Taken together, the above reports suggest that, for some TNFSF/TNFRSF ligand/receptor pairs at least, it is essential to cluster receptors together for full

signaling activity. By this interpretation, the efficacy of the membrane forms of FasL, TNF, and CD40L occurs because these ligands can move in the plane of the membrane toward the contact zone with a receptor-bearing responding cell, thereby clustering ligated receptors to form a receptor-dense region of the membrane. This interpretation is further supported by experiments where crosslinking of a soluble TNFSF protein effectively mimics the activity of the membrane form of the protein [Scheider, 1998].

In all of the above examples, no more than three amino acid chains have been caused to associate together. There is a need to produce multimeric protein molecules where more than three amino acid chains are caused to associate into a single soluble molecular complex. An important example comes from studies of CD40L (also called CD154 or TNFSF5), which is a member of the TNF family of molecules that are normally expressed as insoluble, cell membrane proteins. It has been shown that soluble homotrimers composed of the extracellular regions of CD40L, TNF, and FasL are not potently active on resting cells that bear receptors for these proteins. However, if these proteins are expressed with a tag on their ends (e.g., the FLAG peptide sequence) and then the trimers are extensively crosslinked using an antibody to FLAG, full activity appears (Schneider *et al.*, *J. Exp. Med.* 187:1205, 1998). From this, it can be inferred that the soluble single-trimer forms of these molecules does not duplicate the multivalent interactions that normally occur when a receptor-bearing cell comes in contact with the membrane of a cell expressing numerous ligand trimers on its surface. This distinction may be due to a need for receptor clustering for full signaling (Bazzoni and Beutler, *N. Engl. J. Med.* 334:1717, 1996), which in turn is only possible with a multimeric ligand engaging many receptors at the same time in a localized region of the

cell membrane.

SUMMARY OF THE INVENTION

The present invention contemplates a method of preparing soluble, multimeric mammalian proteins by culturing a host cell transformed or transfected with an expression vector encoding a fusion protein comprising the hub, body, and neck region of a collectin molecule and a heterologous mammalian protein.

In one embodiment, the heterologous mammalian protein comprises an extra cellular domain of a mammalian transmembrane protein; the resulting fusion protein forms a multimer.

In another embodiment, the heterologous mammalian protein comprises a soluble protein such as a cytokine; the resulting fusion protein forms a multimer.

In another embodiment, sites of proteolytic degradation are included or removed from the fusion protein; the resulting fusion protein forms a multimer from which are cleaved single units at a rate made variable by the nature of the proteolytic digestion sites either included or excluded.

In yet another embodiment, special attention is given to the immunogenicity of the fusion protein by altering the junction between the two naturally occurring proteins from which it is made; the resulting fusion protein may be less or more able

to elicit an immune response against itself, which could lengthen its persistence or contribute to it immunological effectiveness.

A hybrid nucleotide sequence of no more than 1528 base pairs including a sequence defining a structural gene expressing a conjoined single strand of a multimeric TNFSF-SPD fusion protein, said structural gene having a nucleotide base sequence selected from members of the group consisting of SEQ ID NO 1, SEQ ID NO 3 and SEQ ID NO 5 is disclosed by this invention. In one embodiment, the DNA segment the structural gene has a sequence expressing a single hybrid amino acid chain of TNFSF-SPD, the segment having a first SPD nucleotide base sequence of SEQ ID NO 1, from base 32 to base 799, and a second sequence, expressing a portion of TNFSF stalk, selected from members of the group consisting of SEQ ID NO 1, from base 800 to base 1444, SEQ ID NO 3, from base 800 to base 1528, and SEQ ID NO 5, from base 800 to base 1441.

In another embodiment, a recombinant DNA molecule has vector operatively linked to an exogenous DNA segment defining a structural gene expressing a single amino acid chain of TNFSF-SPD. This structural gene has a nucleotide base sequence selected from members of the group consisting of SEQ ID NO 1, SEQ ID NO 3 and SEQ ID NO 5, any functional equivalents and modifications thereof There is also attached an appropriate promoter for driving the expression of said structural gene in a compatible host organism. The organism can be E. coli, a yeast, a higher plant or animal.

Yet another embodiment contemplated by the invention is multimeric TNFSF-SPD fusion protein having a plurality of polypeptide trimers, a first trimer consisting of peptide

strands of members of the TNF superfamily (TNFSF) of ligands, and a second trimer strand from a collectin molecule, each first trimer conjoined to a second polypeptide trimer strand from a collectin molecule, wherein said ligand strand is substituted for native carbohydrate recognition domains (CRD) of the collectin molecules. The conjoined collectin strands are covalently bound in parallel to each other, forming a multimeric fusion protein comprising a plurality of trimeric hybrid polypeptide strands radiating from a covalently bound center hub of the molecule. The free end of each trimeric radiating strand has a TNFSF moiety attached. The TNFSF moiety is one selected from the group consisting of ligands LTA, TNF, LTB, and TNFSF4 to TNFSF 18 as shown in Table II, and their funtional equivalents, and modifications thereof.

The invention also contemplates a method for preparing a CD40-SPD multimeric fusion polypeptide, including the steps of initiating a culture, in a nutrient medium, of procaryotic or eucaryotic host cells transformed with a recombinant DNA molecule including an expression vector, appropriate for the cells, operatively linked to an exogenous DNA segment defining a structural gene for CD40-SPD ligand. The structural gene has a nucleotide base sequence of SEQ ID NO 1 from about base 32 to about base 1444. Thereafter, the culture is maintained for a time period sufficient for the cells to express the multimeric molecule.

Also contemplated is a method of producing a secreted, very large, biologically active, multimeric tumor necrosis factor superfamily ligand fusion protein chimera that is highly immunogenic and not readily diffusable. The steps for this method are as follows:

1. introducing into a host cell a first chimeric DNA construct including a

transcriptional promoter operatively linked to a first secretory signal sequence, followed downstream by, and in proper reading frame with a first DNA sequence encoding a polypeptide chain of a first TNFSF ligand requiring multimerization for biological activity. This sequence is joined to a second DNA sequence encoding a collectin polypeptide at the site where the collectin's CRD was purposefully removed.

2. introducing into the host cell, a second DNA construct including a transcriptional promoter operably linked to a second secretory signal sequence followed downstream by, and in proper reading frame with, a third DNA sequence encoding a second polypeptide chain of a second TNFSF ligand , joined to a fourth DNA sequence encoding a collectin polypeptide , wherein the collectin's CRD was purposefully removed, and then,

3. growing the host cell in an appropriate growth medium under physiological conditions to allow the secretion of a large multimerized polypeptide fusion protein, wherein the first polypeptide chain of a TNFSF-SPD protein is bound by parallel bonding of the respective collectin domain trimer to the second polypeptide chain of a different TNFSF-SPD polypeptide trimer, and wherein the multimerized polypeptide fusion protein exhibits biological activity characteristic of both membrane-attached TNFSFs, and

4. isolating the biologically active, multimerized TNFSF-SPD polypeptide fusion from said host cell. The chimeric reactant compounds are humanized to guard against destruction by a potential human recipient's immune system.

A final method of preparing a multimeric TNFSF-SPD ligand fusion protein contemplated requires a) preparing a first DNA segment coding for a strand of an exposed extracellular portion of TNFSF; b) preparing a second DNA segment coding for a collectin polypeptide strand, wherein the collectin's CRD domain of the strand has been removed; c)

conjoining the first and second DNAs in proper reading frame, thereby creating a TNFSF-collectin DNA construct; d) inserting the construct into an expression vector system; e) introducing the vector system into an appropriate cell in culture under suitable conditions; f) harvesting and purifying spent medium from the culture; and finally g) assaying for presence of multimeric TNFSF-collectin fusion protein.

A method for stimulating the immune response in potentially immonocompetent cells using multimeric TNFSF fusion proteins by contacting the cells with the multimeric TNFSF fusion proteins, causing the cells to proliferate, is also contemplated. The cells used may be resting B cells. There is also a method for increasing antigenicity of cells by contacting the cells with the multimeric TNFSF fusion proteins. In this case, the cells may be tumor cells or HIV positive cells.

Other preferred embodiments contemplate the methods of preparation described above, wherein the host transformed is either a prokaryote, such as E. coli, a eukaryote, for example yeast, such as S. cerevisiae, or a higher plant, such as alfalfa or tobacco.

Still further embodiments and advantages of the invention will become apparent to those skilled in the art upon reading the entire disclosure contained herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Structure of the CD40L-SPD fusion protein. The extracellular portion of the CD40L homotrimer, including its membrane-proximal stalk, was fused to the body of SPD. The N-terminus of SPD contains two cysteines which link the homopolymer together by disulfide bonds forming a hub. The trimeric collagenous stalk extend from the hub as a cruciate structure and end in a spontaneously trimerizing neck region. The amino acid domains in a single chain of the CD40L-SPD are shown at the top. At the bottom is the tetrameric (four CD40L trimers) which is expected to form. In addition, the hub region of SPD can participate in stacking up to 8 or more cruciate forms into higher order aggregates.

Figure 2. Ion-exchange chromatography of murine CD40L-SPD. CHO cells expressing murine CD40L-SPD were grown in serum-free media, concentrated using a 100 kDa cutoff ultrafiltration membrane, and diafiltered into 50 mM bicine, pH 9.0, 1 mM EDTA. Using an FPLC system, the protein from 400 mL of media was applied to a Fractogel SO_3^- 650M column and eluted with a linear salt gradient. 3 mL samples were collected. Shown are curves for protein concentration (OD_{280}), conductivity as % 1 M NaCl in the buffer, and ELISA-detectable CD40L-SPD assayed at 1:100 dilution.

Figure 3. Size fractionation of murine CD40L-SPD by ultrafiltration. CD40L-SPD is a 471 amino acid protein with a predicted molecular weight of 49,012 for each of the twelve component chains in the dodecamer (composed of four trimeric subunits). This does not include added carbohydrates. Therefore, the full dodecamer will have a molecular weight in excess of 600,000. However, from the literature on recombinant surfactant protein D made

in CHO cells, it appears that some of the product will be in the form of trimers that are not part of a cruciate-formed dodecamer. To determine what percentage of CD40L-SPD was produced in a multimeric form, supernatant from the transfected CHO cells were passed through filters of different porosities (rated for their ability to retard globular proteins). An ELISA was used to detect the amount of CD40L-SPD (measured at multiple dilutions) that passed through the filter. As shown, about 90% of the protein is retained by a 300,000 kDa cut-off filter. This indicates that most of the protein is in the dodecameric form. In addition, the cruciate dodecamers of surfactant protein D can also stack on top of each other into even higher molecular weight forms. This is the likely explanation for the small fraction of CD40L-SPD that is retained by the 1,000 kDa cut-off filter.

Figure 4. Activation of human B cells by human CD40L-SPD. Conditioned media from CHO cells expressing human CD40L-SPD was added to human B cells along with IL-4. In the left panel, the cells were stained with CyChrome-labeled anti-CD19 to identify B cells and PE-labeled anti-CD3 to identify T cells. As shown, most of the cells proliferating in the culture were CD19+ CD3- B cells. In the right panel, the cells were stained with CyChrome-labeled anti-CD19 to identify B cells and PE-labeled anti-CD80 (B7-1) to identify this co-stimulatory molecule. As shown, almost all of the B cells were induced by CD40L-SPD to express CD80.

Figure 5. Activation of murine B cells by murine CD40L-SPD. Murine CD40L-SPD was added to resting murine splenic B cells for a two day culture period. For the final 4 hours, the cultures were pulsed with ^{3}H-thymidine, following which the cells were harvested and DNA synthesis was measured by scintillation counting. As shown, CD40L-SPD is

nearly as effective as anti-IgM in promoting the proliferation of resting B cells.

Figure 6. CD40L-SPD stimulation of macrophage chemokine production. Conditioned media from CHO cells expressing human CD40L-SPD, an inactive mutant of human CD40L-SPD (T147N-CD40L-SPD), or murine CD40L-SPD (mCD40L-SPD) were added to cultures of human monocyte-derived macrophages. As a negative control, this media was heat-inactivated at 60 °C for 30 minutes. Also shown is a form of soluble CD40L (sCD40L) consisting of 149 amino acids from the extracellular domain of human CD40L (Peprotech) added at 1 ?g/mL. 24 hours later, supernatants were collected and assay for MIP-1? by ELISA (R & D Systems). The weak activity of soluble single-trimer CD40L (sCD40L) is apparent. In contrast, native human and murine CD40L-SPD strongly activated the macrophages to produce MIP-1?. In contrast, heat-inactivated CD40L-SPD was inactive. As expected, the inactive mutant, T147N-CD40L-SPD, also failed to stimulate macrophages, demonstrating that the CD40L portion and not the SPD portion of the protein was responsible for stimulating the macrophages.

Figure 7. Expression of RANKL/TRANCE-SPD production from CHO cells detected by ELISA. Antibodies against RANKL/TRANCE were used to construct an ELISA capable of detecting the RANKL/TRANCE protein. As shown, there was no background with the media control. Using a fusion protein between CD70 (CD27L or TNFSF7) and SPD, there was also no signal, indicating the specificity of the ELISA. However, using CHO cells transfected with an expression plasmid for CD70-SPD, immunoreactive secreted protein was clearly detectable. This demonstrates the generalizability of the method for expressing TNFSF members as fusion proteins with collectins such as SPD.

DESCRIPTION OF THE PREFERRED EMBODIMENT

1. Definition of Terms

Multimeric: As used herein the term multimeric refers to a multimer of a polypeptide that is itself a trimer (i.e., a plurality of trimers).

Functional Equivalent: Herein refers to a sequence of a peptide or polypeptide that has substantial structural similarity and functional similarity to another such sequence.

Modifications: Herein refers to point changes involving single amino acids, wherein the functionality is altered, without appreciably altering the primary sequence or primary structure of a peptide or polypeptide.

Amino Acid: All amino acid residues identified herein are in the natural L-configuration. In keeping with standard polypeptide nomenclature, J. Biol. Chem., 243:3557-59, (1969), abbreviations for amino acid residues are as shown in the following Table of Correspondence:

TABLE OF CORRESPONDENCE

SYMBOL		AMINO ACID
1-Letter	3-Letter	
Y	Tyr	L-tyrosine
G	Gly	glycine
F	Phe	L-phenylalanine
M	Met	L-methionine
A	Ala	L-alanine
S	Ser	L-serine
L	Ile	L-isoleucine
L	Leu	L-leucine
T	Thr	L-threonine
V	Val	L-valine
P	Pro	L-proline
K	Lys	L-lysine
H	His	L-histidine
Q	Gln	L-glutamine
E	Glu	L-glutamic acid
W	Trp	L-tryptophan
R	Arg	L-arginine
D	Asp	L-aspartic acid
N	Asn	L-asparagin
C	Cys	L-cysteine

It should be noted that all amino acid residue sequences are represented herein by formulae whose left to right orientation is in the conventional direction of amino-terminus to carboxy-terminus. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a bond to a radical such as H and OH (hydrogen and hydroxyl) at the amino- and carboxy-termini, respectively, or a further sequence of one or more amino acid residues up to a total of

about fifty residues in the polypeptide chain.

Base Pair (bp): A partnership of adenine (A) with thymine (T), or of cytosine (C) with guanine (G) in a double stranded DNA molecule.

Constitutive promoter: A promoter where the rate of RNA polymerase binding and initiation is approximately constant and relatively independent of external stimuli. Examples of constitutive promoters include the cauliflower mosaic virus 35S and 19S promoters described by Poszkowski et al., *EMBO J.*, 3:2719 (1989) and Odell et al., *Nature*, 313:810 (1985).

DNA: Desoxyribonucleic acid.

Enzyme: A protein, polypeptide, peptide RNA molecule, or multimeric protein capable of accelerating or producing by catalytic action some change in a substrate for which it is often specific.

Expression vector: A DNA sequence that forms control elements that regulate expression of structural genes when operatively linked to those genes.

Expression: The combination of intracellular processes, including transcription and translation undergone by a structural gene to produce a polypeptide.

Insert: A DNA sequence foreign to the rDNA, consisting of a structural gene and optionally additional DNA sequences.

Nucleotide: A monomeric unit of DNA or RNA consisting of a sugar moiety (pentose), a phosphate, and a nitrogenous heterocyclic base. The base is linked to the sugar moiety via the glycosidic carbon (1' carbon of the pentose) and that combination of base and sugar is a nucleoside. When the nucleoside contains a phosphate group bonded to the 3' or 5' position of the pentose it is referred to as a nucleotide.

Operatively linked or inserted: A structural gene is covalently bonded in correct reading frame to another DNA (or RNA as appropriate) segment, such as to an expression vector so that the structural gene is under the control of the expression vector.

Polypeptide and peptide: A linear series of amino acid residues connected one to the other by peptide bonds between the alpha-amino and carboxy groups of adjacent residues.

Promoter: A recognition site on a DNA sequence or group of DNA sequences that provide an expression control element for a gene and to which RNA polymerase specifically binds and initiates RNA synthesis (transcription) of that gene.

Inducible promoter: A promoter where the rate of RNA polymerase binding and initiation is modulated by external stimuli. Such stimuli include light, heat, anaerobic stress, alteration in nutrient conditions, presence or absence of a metabolite, presence of a ligand, microbial attack, wounding and the like.

Spatially regulated promoter: A promoter where the rate of RNA polymerase binding and initiation is modulated in a specific structure of the organism such as the leaf, stem or root. Examples of spatially regulated promoters are given in Chua et al., *Science*, 244:174-181 (1989).

Spatiotemporally regulated promoter: A promoter where the rate of RNA polymerase binding and initiation is modulated in a specific structure of the organism at a specific time during development. A typical spatiotemporally regulated promoter is the EPSP synthase-35S promoter described by Chua et al., *Science*, 244:174-181 (1989).

Temporally regulated promoter: A promoter where the rate of RNA polymerase binding and initiation is modulated at a specific time during development. Examples of temporally regulated promoters are given in Chua et al., *Science*, 244:174-181 (1989).

Protein: A linear series of greater than about 50 amino acid residues connected one to the other as in a polypeptide.

Recombinant DNA molecule: A hybrid DNA sequence comprising at least two nucleotide sequences not normally found together in nature.

RNA: Ribonucleic acid.

Selective Genetic marker: A DNA sequence coding for a phenotypical trait by means of which transformed cells can be selected from untransformed cells.

Structural gene: A DNA sequence that is expressed as a polypeptide, i.e., an amino acid residue sequence.

Synthetic promoter: A promoter that was chemically synthesized rather than biologically derived. Usually synthetic promoters incorporate sequence changes that optimize the efficiency of RNA polymerase initiation.

2. Introduction

This invention discloses the production of TNFSF proteins as multimeric (i.e., many trimers) ligands fused onto a trimeric, branched protein backbone. Collectin molecules are ideal for this purpose because they are formed from many trimeric, collagenous arms linked to a central hub by disulfide bonds. Of the collectins, pulmonary surfactant protein D (SPD) was chosen initially because it is a homopolymer encoded by a single gene, unlike C1q and surfactant protein A, which are composed of two different protein subunits. In addition,

recombinant SPD has been successfully expressed *in vitro* in reasonable yield [Crouch, 1994], and a peptide containing the "neck" region of SPD was shown to spontaneously trimerize in solution [Hoppe, 1994]. Consequently, extracellular domains of human and murine CD40L were substituted for the carbohydrate recognition domain of pulmonary surfactant D (SPD) to create a four-armed molecule (three peptide chains per arm) with CD40L at the end of each arm. This molecule is named CD40L-SPD. In addition, because SPD tends to stack into higher order aggregates with up to 8 molecules associated at the hub [Crouch], even greater degree of multimerization can occur [Lu, 1993]. CD40L-SPD therefore mimics the expression of CD40L by an activated T cell in that it presents a multivalent complex similar to membrane-bound CD40L. While remaining soluble, CD40L-SPD equals membrane CD40L in its range of activities.

3. Construction of expression plasmids for CD40L-SPD.

cDNAs of exposed human and murine CD40L, removed from cell membranes, were cloned by PCR by well-known methods. Murine surfactant protein D was cloned by hemi-nested PCR from murine lung mRNA (Clonetech). cDNA was prepared using Superscript II reverse transcriptase (Life Technologies, Gaithersburg, MD) and random hexamers as primers. PCR primer sequences were as follows (the underlined bases indicate restriction endonuclease sites for cloning into the vector):

mSPD5: 5'-CTGACATGCTGCCCTTTCTCTCCATGC-3'

mSPD3ext: 5'-GGAGGCCAGCTGTCCTCCAGCCTGTTTGC-3'

rmSPD5: 5'-GGGG<u>'CTAGC</u>**GAATTCCACCAGGAAGCAAT**CTGACATGCTGCCCTTT-CTCTCCATGC-3'

CD40L/SPD3: 5'-TCTATCTTGTCCAACCTTCTATG/GCCATCAGGGAACAATGCAGCTTTC-3'

SPD/CD40L5: 5'-AAAGCTGCATTGTTCCCTGATGGC/CATAGAAGGTTGGACAAGATAGAAG-3'

CD40L3: 5'-GGGCTCGA<u>GGTACC</u>AGTTCTACATGCCTTGGAGTGTATAAT-3'

SPD/mCD40L5: 5'-GAAAGCTGCATTGTTCCCTGATGGC/CATAGAAGATTGGATAAGGTCGAAG-3'

mCD40L/SPD3: 5'-CTTCGACCTTATCCAATCTTCTATG/GCCATCAGGGAACAATGCAGCTTTC-3'

mCD40L3: 5'-GGGG<u>GGTACC</u>CTGCTGCAGCCTAGGACAGCGCAC-3'

Because the murine SPD sequence of the 5' untranslated region containing the ribosomal binding site was unknown when this work was started [Motwani, 1995], a primer (rmSPD5) was designed based on the available rat sequence [Shimizu, 1992] which extended the 5' end with rat sequence (shown in bold) along with an added Nhe I site (underlined).

4. <u>Creation of the CD40L-SPD Fusions</u>.

To create the CD40L-SPD fusions, overlap PCR was used. Murine SPD was amplified by nested PCR using mSPD5 and mSPD3ext for the first round of 30 cycles. The product was diluted 1:1,000 and 1 µL was amplified for another 30 cycles using rmSPD5 and CD40L/SPD3, where the 3' half of CD40L/SPD3 is a reverse primer for SPD C-terminal to the neck region (deleting the CRD) and the 5' half of CD40L/SPD3 contains bases from the N-terminus of the extracellular portion of CD40L (immediately adjacent to the transmembrane region). Similarly, the CD40L plasmid was amplified with SPD/CD40L5 and CD40L3, which contains a

Kpn I site (underlined). All of these PCRs were performed with Pfu cloned polymerase (Stratagene,) using hot start (Ampliwax, Perkin-Elmer) and the thermocycling program: 94 °C for 2.5 min; then 30 cycles of 94 °C for 10 sec, 43 °C for 30 sec, and 75 °C for 7 min.

To form the chimeric construct, 1 μL of a 1:1,000 dilution of gel-purified products from the above reactions was combined and amplified with rmSPD5 and CD40L3. Because Pfu polymerase did not consistently yield the expected 1.62 kb overlap product, AccuTaq LA DNA polymerase (Sigma) was used for this PCR, using the thermocycling program: 94 °C for 2.5 min; then 30 cycles of 98 °C for 20 sec, 43 °C for 30 sec, and 68 °C for 10 min. The resulting product was digested with Nhe I and Kpn I, gel-purified, and ligated into the Nhe I and Kpn I sites in the expression plasmid, pcDNA3.1(+) (Invitrogen, Carlsbad, CA). DH5 E. coli were transformed with the construct and plasmid DNA was purified either by double banding in ethidium bromide-CsCl gradients or by anion exchange resin (QIAgen). To form the T147N-CD40L-SPD construct, the same approach was used except that the CD40L coding region was taken from the expression plasmid for T147N-CD40L [Kornbluth]. The amino acid sequence at the junction between SPD and CD40L is ...KAALFPDG/HRRLDKIE..., where the C-terminal portion begins the sequence for CD40L. To form mCD40L-SPD, a similar approach was taken except that primers SPD/mCD40L5, mCD40L/SPD3, and mCD40L3 were used for amplifications involving murine CD40L sequences. The amino acid sequence at the junction between SPD and murine CD40L is ...KAALFPDG/HRRLDKVE..., where the C-terminal portion begins the sequence for murine CD40L. Both DNA strands of each construct were sequenced to confirm that the constructs were correct. In other experiments, an entirely humanized construct, consisting of human CD40L fused to

human SPD, was constructed (data not shown).

5. Construction of expression plasmid for murine RANKL/TRANCE (TNFSF 11). Spleen cells from C3H/HeJ mice were stimulated with 5 μg/ml concanavalin A and 10 ng/ml IL-2 (Sigma) for 8 hours (31). mRNA was isolated using the Micro FastTrack kit (Invitrogen). cDNA was prepared using Superscript II reverse transcriptase (Life Technologies) and random hexamers as primers. PCR primer sequences were as follows (where the underlined bases indicate restriction endonuclease sites for cloning into the vector):

5mRANKL-ext:	5'-CATGTTCCTGGCCCTCCTC-3'
3mRANKL-ext:	5'-GTACAGGCTCAAGAGAGAGGGC-3'
5mRANKL-int:	5'-ATACTCGAGCGCAGATGGATCCTAAC-3'
3mRANKL-int:	5'-GGGGTTTAGCGGCCGCTAATGTTCCACGAAATGAGTTC-3'

The extracellular portion of RANKL/TRANCE was cloned by nested PCR. In the first round of PCR, 5mRANKL-ext and 3mRANKL-ext were used with Pfu cloned polymerase (Stragene) using the thermocycling program: 94 °C for 2.5 min; then 30 cycles of 94 °C for 10 sec, 50 °C for 30 sec, and 75 °C for 2 min. The product was diluted 1:1,000 and 1 μL was amplified for another 30 cycles using 5mRANKL-int and 3mRANK-int, which contain an Xho I site and a Not I site respectively. The resulting product was digested with Xho I, blunt-ended with T4 DNA polymerase, then digested with Not I and gel-purified. The CD40L-SPD expression plasmid described above was digested with Msc I and Not I and gel purified. Then the RANKL/TRANCE sequence was ligated into this vector in frame with the SPD coding sequence. The amino acid sequence at the junction between SPD and RANKL/TRANCE is ...KAALFPDG/RAQMDPNR..., where the N-terminal portion is from SPD and the C-terminal portion is the extracellular sequence of

RANKL/TRANCE. Both DNA strands of each construct were sequenced to confirm that the constructs were correct.

6. **Stable transfection of DHFR-deficient CHO cells and amplification.**

DG44 (a line of CHO-K1 cells deficient in dihydrofolate reductase (DHFR)) (32) and pCH1P (a plasmid containing the hamster DHFR minigene) (33) were gifts from Dr. Lawrence Chasin, Columbia University, New York, NY . DG44 cells were cultured in α-MEM consisting of ribo- and deoxynucleoside-free α-MEM (BioWhittaker, Walkersville, MD) supplemented with 200 μM L-glutamine, 10% fetal bovine serum (FBS) and 10 μg/ml each of adenosine, deoxyadenosine, and thymidine (Sigma). All cell cultures described were negative in a mycoplasma rRNA assay (Gen-Probe, San Diego). DG44 cells in six-well plates were transfected by the method of Okayama and Chen ((34) with 10 μg of expression plasmid and 0.05 μg of pCH1P (200:1 ratio). After two days, the transfected DG44 were trypsinized and transferred to 100 mm plates. At this point, the media was switched to α$^-$-MEM which differs from α-MEM in that dialyzed FBS (HyClone Systems, Logan, UT) was used and no nucleoside supplements were added. Only cells containing the DHFR minigene were able to grow in α$^-$-MEM, and colonies were selected after 10 days, cloned using cloning rings, and transferred to 12.5 cm^2 flasks. Clones were selected for expansion using an ELISA to screen for the production of either murine or human CD40L (see below). Using the method described by Kingston *et al.* (35), escalating doses of methotrexate were used to amplify the transfected genes over a period of 6-14 months until the cells grew well in 80 μM methotrexate. Each expressing clone was re-cloned once or twice more in order to select the highest expressing cells.

7. **Preparation of human and murine CD40L-SPD in serum-free media.**

Selected clones were adapted for growth in nucleoside-free UltraCHO media

(BioWhittaker) supplemented with 50-100 μg/mL ascorbic acid and 50 μM methotrexate (Sigma). The non-adherent population was further adapted for suspension growth in roller bottles. In some experiments, the cells were adapted from α-MEM to CHO-S-SFM II media (Life Technologies) supplemented with ascorbic acid and 50 μg/mL L-proline.

8. ELISA assay for human and murine CD40L-SPD.

To assay for correctly folded CD40L, wells of a MaxiSorb 96-well plate (Nunc) were coated overnight at 4 °C with 50 μL of carbonate-bicarbonate, pH 9.40 buffer containing 0.5 μg/mL 24-31 anti-human CD40L MAb (Ancell) or MR1 anti-murine MAb (Bioexpress, Lebanon, NH). Wells were blocked with 3% bovine serum albumin (BSA) in PBS. 100 μL samples were added to the wells either neat or diluted in a dilution buffer consisting of 1% BSA, 0.9 % NaCl, 50 mM Tris pH 7.40, and 0.1% peroxide-free Tween 20 (Sigma). After shaking for 2 h at 600 RPM, a plate washer was used to wash the plate four times with 0.9 % NaCl, 50 mM Tris pH 7.40, and 0.1% peroxide-free Tween 20. Then, 100 μL of diluent buffer containing 1 μg/mL biotinylated 24-31 anti-human CD40L Mab (Ancell) or MR1 anti-murine CD40L Mab (Pharmingen, San Diego, CA) was added to each well and again shaken for 2 h. Following another four washes, 100 μL of diluent buffer containing 1 μg/mL of streptavidin-alkaline phosphatase (Jackson) was added to each well and the plate was shaken for 1 hour. Lastly, after another four washes, color was developed for 10-20 min using 100 μL/well of BluePhos (Kierkegaard & Perry), stop solution was added, and the wells were read at 650 μm in a plate reader.

9. Purification of human and murine CD40L-SPD.

Conditioned UltraCHO media was filtered using a 0.2 μ PES filter unit (Nalgene) and stored at 4 °C for up to 3 months. A preliminary size fractionation

was performed by ultrafiltration through a 100 kDa-cutoff 76 mm membrane (YM-100, Millipore) in a 400 mL stirred cell at 10 lbs/sq. inch pressure of argon. Media was concentrated to about 10 mL, diluted to 100 mL with buffer, and again concentrated to 10 mL for a total of 3 cycles of ultrafiltration and buffer exchange. Buffer was 50 mM Bicine (Calbiochem), adjusted to pH 9.0 with NaOH (about 32 mM Na), and 1 mM EDTA to prevent the activity of any metalloproteinase. Using FPLC equipment (Amersham-Pharmacia), the concentrate was filtered through a 0.45 μ filter, placed into a 10 mL superloop, applied to a 10 X 30 mm column (HR10/30, Amersham-Pharmacia) packed with Fractogel SO_3^- 650M (EM Biosciences), and eluted at 0.5 mL/min at 4 °C with a linear gradient of 0-500 mM NaCl in buffer. As described by the manufacturer, the resolution of proteins on Fractogel SO_3^- is enhanced by using a long, thin column geometry. Fractions were collected and screened for human or murine CD40L by ELISA. Positive fractions were pooled, concentrated by ultrafiltration (CentriPrep-30, Millipore), filtered through a 0.45 μ filter, and applied to a Superose 6 column (Amersham-Pharmacia) in phosphate-buffered saline.

10. Murine B cell cultures.

C3H/HeJ mice were euthanized by CO_2 inhalation under a protocol approved by the Animal Subjects Committee of the San Diego VA Healthcare System. Splenocytes were isolated by centrifugation over Lympholyte-M (Accurate Chemical & Scientific Corp., Westbury, NY) and B cells were isolated by negative selection using anti-CD43 immunomagnetic beads (Miltenyi Biotec Inc., Auburn, CA). The resting B cells were suspended in Dulbecco's MEM with 10% FBS at a concentration of 1 X 10^6/mL, and 100 μL was added to the wells of 96-well flat-bottomed plates. 100 μL of dilutions of murine CD40L-SPD in media or media alone were added to the wells, which were incubated in

8.5% CO_2 at 37 °C for 48 hours. Then, 0.5 μCi/well of ^{3}H-thymidine was added to each well, and the cells were collected 4 h later onto glass fiber filters using an automated cell harvester. A scintillation counter was used to determine the incorporated radioactivity.

11. **Human B cell cultures**.

Venous blood from consenting subjects was used as a source of human B cells under a protocol approved by the UCSD Institutional Review Board. Blood was collected into syringes containing 5 U/mL heparin and peripheral blood mononuclear cells (PBMC) were isolated by centrifugation over Ficoll-hypaque. The cells were suspended at 2 X 10^5/mL in RPMI 1640 containing 200 μM L-glutamine, 10% FBS, 0.832 μM cyclosporin A (Sigma), and 25 ng/mL human IL-4 (R & D Systems) and incubated in 5% CO_2 at 37 °C as described by Schultze *et al.* (36). At intervals, the cells were stained with CyChrome-conjugated anti-CD19 and PE-conjugated anti-CD80 (B7-1) monoclonal antibodies (Pharmingen) and analyzed by flow cytometry.

12. **Human monocyte-derived macrophage and dendritic cell cultures.**

As previously described [Kornbluth], monocytes were isolated from PBMC by adherence to fibronectin-coated plates, plated into 48-well plates, and then cultured in RPMI1640 containing 200 ?M L-glutamine and 10% autologous serum for 7-10 days. Monolayers of the matured cells (about 2 X 10^5/well), termed monocyte-derived macrophages or MDM, were then washed in media and cultured in 1 mL/well RPMI1640 containing 200 ?M L-glutamine and 10% heat-inactivated FBS. Alternatively, dendritic cells (DC) were formed from monocytes by adding GM-CSF and IL-4 to the culture media, and the resulting DC were used 6 days later. Preparations of CD40L-SPD were added to the wells as indicated. As a positive control, 100 ng/mL bacterial lipopolysaccharide (LPS) from E. coli 0111:B4

(Calbiochem) was added. Supernatants were collected 24 h later and analyzed for cytokine content using ELISA (R & D Systems).

Example 1.

Design principles in constructing collectin-TNFSF member fusion proteins.

To express CD40L and other TNFSF members as stable, multimeric proteins, the coding region of the extracellular, C-terminal portion of CD40L was joined in-frame to the collectin, surfactant protein D (SPD). The N-terminus of SPD contains two cysteines which form the disulfide bonds necessary for the 4-armed cruciate structure of the overall molecule [Brown-Augsburger, 1996~~#506~~]. C-terminal to these cysteines in SPD is a long triple-helical collagenous "stalk" which ends in the "neck" region that promotes the trimerization of each arm of the structure. Immediately after this neck region, the coding sequence for the extracellular portion of CD40L was added, in place of the carbohydrate recognition domain (CRD) of SPD. The collectins were chosen as the framework for the multimeric construct because of their multi-subunit structure and the trimeric nature of their stalk regions. Appropriateness of replacing the CRD of a collectin with the extracellular region of a TNFSF member is further supported by structural studies of the two protein families. An analysis of the CRD crystal structure of another collectin, ACRP30, indicated that it was structurally superimposable upon the crystal structures of the extracellular regions of CD40L, TNF, and Fas [Shapiro, 1998]. The successful expression of the collectin-TNFSF fusion protein, CD40L-SPD, indicates that other TNFSF members (Table I) could be conjoined to SPD in a similar manner and that other collectins besides SPD (Table II) could be used as a protein framework instead of SPD. Because these molecules are formed entirely from naturally occurring proteins, the production of an immune response (e.g., antibodies) to these fusion proteins is

minimized. By deleting portions of the stalk region of the TNFSF proteins, additional constructs can be made which may be even less immunogenic.

Example 2.

Expression of human and murine CD40L-SPD in CHO cells.

The coding regions for the extracellular portion of human CD40L, human T147N-CD40L ,an inactive mutant of CD40L, or murine CD40L were joined to the neck region of murine SPD, replacing the SPD CRD (Fig. 1). A CMV-driven expression plasmid for the construct was co-transfected with a DHFR minigene into DNFR-deficient CHO cells. Following selection in nucleoside-free media, expressing CHO clones were amplified by culture in ascending doses of methotrexate. The resulting clones produced about 1-10 μg/mL of the fusion protein over a 3 day period in media containing FBS.

Clones were adapted for growth as suspension cells in two types of serum-free media. Murine CHO-SPD produced in UltraCHO (BioWhittaker) was largely retained (about 60% as determined by ELISA) by a 1,000 kDa cutoff ultrafiltration membrane (Pall Corp., Port Washington, NY), consistent with a large multimeric complex formed by the stacking of the SPD portion of the molecule. However, in CHO-S-SFM II (Life Technologies), nearly all ELISA-detectable murine CHO-SPD passed through a 100 kDa cutoff ultrafiltration membrane (Millipore), suggesting that the protein was either folding incorrectly in this media or was being degraded by proteolysis. Consequently, the purification method was optimized for the spent UltraCHO media.

Example 3.

Purification of human and murine CD40L-SPD.

Purification procedures were developed for murine CD40L-SPD, but the same methods could be applied to human CD40L-SPD with minor modifications. Murine CD40L-SPD has a predicted m.w. of 49 kDa per chain, or about 600 kDa per 12-chain, cruciate molecule, the amino acid sequence predicts a pI of 9.10. Accordingly, conditioned media was concentrated by ultrafiltration through a 100 kDa cutoff filter, which also fractionates the sample on a size basis. After diafiltration into 50 mM bicine, pH 9.00 (also containing 1 mM EDTA added to inhibit metalloproteinases), the sample was applied to a variety of cationic exchange resins. Using Source 30S (Amersham-Pharmacia), most of the ELISA-detectable protein did not bind and was recovered in the flow-through. However, as reported by Morris et al. {Morris}, Fractogel SO_3 650M retained the protein. The retention by this tentacular resin and not by Source 30S suggests binding to positively charged residues that are not on the protein surface. Using a linear NaCl gradient, ELISA-detectable protein elutes at between 0.15-0.30 M NaCl under these conditions (Fig. 2). In selected experiments, the protein was further purified using a Superose 6 sizing column. Most of the ELISA-detectable protein eluted in the excluded volume, indicating an apparent m.w. of greater than 1,000 kDa (Fig. 3).

Example 4.

Activity of CD40L-SPD on human B cells.

Schultze *et al.* described a system using CD40L-expressing cells plus IL-4 and cyclosporin A (to inhibit T cell growth) as a means to grow very large numbers of B cells from a small sample of blood. Because CD40L activates these B cells to express high levels of B7 molecules (CD80 and CD86), the proliferating B cells were effective in presenting

peptide antigens and rival non-dividing dendritic cells as antigen-presenting cells (APCs) (36). To determine if the CD40L-SPD fusion protein could replace CD40L-expressing cells in this system, PBMC were cultured with CD40L-SPD in addition to IL-4 and cyclosporin A. Under these conditions the cells grew to saturation density every three days. After three weeks, the cultures were almost entirely CD19+ B cells which express high levels of CD80 (Fig. 4). This indicates that CD40L-SPD can be used in *ex vivo* systems where a soluble yet effective form of CD40L is needed to stimulate cells for immunotherapeutic applications.

Example 5.

Activity of CD40L-SPD on murine B cells.

Resting murine B cells are particularly difficult to stimulate with most soluble forms of CD40L. Even with murine CD40L-CD8 fusion proteins, it is necessary to crosslink the protein with antibodies against CD8 in order to achieve maximal proliferation in culture [Klauss, 1999]. Accordingly, resting murine B cells were negatively selected with immunomagnetic beads. As shown in Fig. 5, murine CD40L-SPD was as effective as anti-IgM antibody in driving B cells to proliferate. This indicates that CD40L-SPD can mimic the multivalent interactions that occur when a responding cell comes in contact with CD40L-bearing activating cells.

Example 6.

Activity of CD40L-SPD on human macrophages and dendritic cells.

CD40L is a powerful stimulant for macrophages (reviewed in (28)) and dendritic cells (40). Accordingly, preparations of CD40L-SPD were added to monocyte-derived macrophages and the production of MIP-1β was used as a measure of stimulation. As shown in Fig. 6, both human and murine CD40L-SPD were able to stimulate macrophages, whereas the T147N-CD40L-SPD mutant was inactive as expected.

DISCUSSION

These examples define a new method of producing multimeric (i.e., many trimers) of CD40L as a fusion protein with SPD. Also prepared and expressed were similar fusion proteins between murine RANKL/TRANCE (TNFSF11) or murine CD27L/CD70 (TNFSF7) joined to murine SPD (data not shown). This suggests that virtually all TNFSF members could be successfully produced as fusion proteins with SPD. Furthermore, it is also likely that other collectins besides SPD could be used in these fusions, given the strong structural homologies between the CRDs of the collectins and the extracellular domains of TNFSF members [Shapiro] which can be substituted for these CRDs. Given the 17 known TNFSF members and 9 known collectins, at least 153 fusion protein combinations are possible.

SPD was selected for initially because it is a soluble homopolymer. Other collectins, such as surfactant protein A, have strong binding affinities to lipids and specific cell receptors. Although removal of the CRD abrogates much of this binding, it may be partially mediated by the neck region sequence, which the fusion proteins retain. Accordingly, it would be expected that collectins other than SPD might confer different cell-binding and pharmacokinetic behaviors upon a fusion protein. For example, macrophages are known to take up and degrade whole SPD [Dong, 1998]. If a fusion protein other than SPD were used, the disposition of the fusion protein *in vivo* might be altered. Additionally, metalloproteinases are known to degrade the collectin, C1q , so that a fusion with C1q may alter the degradation of the fusion protein. For example, because CD40L activates macrophages and other cells to produce metalloproteinases, which could potentially degrade the collagenous portion of SPD and other collectins. Cleavage of the collagenous stalk would then be expected to release single-trimers of CD40L, which could diffuse away from the

original parent molecule, much like a slow-release formulation of a drug. Also, the membrane-proximal portion of CD40L has been retained in CD40L-SPD. This sequence also contains protease-susceptible amino acid sequences, which can be eliminated by mutagenesis to retard the cleavage of CD40L from the fusion protein. Mutations in such proteinase cleavage site(s) would delay such cleavage and favor the local persistence of the CD40L stimulus.

CD40L-SPD is a large macromolecule (> 1,000 kDa), and the other TNFSF-collectin fusion proteins would be expected to be similarly large. For native SPD, the aggregates that spontaneously form measure 100 nm in diameter. When injected into tissue, this large a complex would be expected to remain at the injection site for a prolonged period. Localization of the TNFSF-containing protein would also be expected to reduce any systemic toxicity caused by the release of free single-trimers into the circulation. For example, soluble CD40L in blood has been linked to disease activity in lupus, and this smaller molecule may even cross the glomerulus to cause damage to renal tubules [Kato and Kipps, J. Clin. Invest. Nov. 1999]. On the other hand, because CD40L induces the production of chemokines which attract immune cells [Kornbluth], T cells, monocytes, and dendritic cells would be expected migrate to the site where CD40L-SPD was injected. This might be advantageous if CD40L-SPD were used as a vaccine adjuvant. In mice, soluble CD40L (sCD40LT) stimulates IgG1 production but not cytotoxic T lymphocytes (CTLs) [Wong, 1999]. Interestingly, the same protein that is expressed from an injected plasmid stimulates both a strong antibody and CTL response [Gurunathan, 1998]. In the latter case, the plasmid would be expected to deliver a localized supply of CD40L, whereas the sCD40LT protein is free to diffuse away. Support for the localized use of CD40L in an adjuvant formulation is provided by a study using a plasmid expressing full-length

membrane CD40L, which was very effective in stimulating both humoral and CTL immune responses [Mendoza, 1997]. Similarly, injection of adenovirus expressing membrane CD40L has potent antitumor activity in mice [Kikuchi, 1999]. Similar considerations would likely apply to other fusion proteins between the TNFSF and collectins.

Finally, for immunostimulatory proteins, it is particularly important that the protein not be antigenic if repeated injections are needed. For example, vaccination with TNF-μ modified by the addition of short peptide sequences was able to induce the production of disease-modifying anti-TNF-μ autoantibodies [Dalum, 1999]. Because CD40L-SPD and other TNFSF-collectin fusion proteins are formed from endogenous protein sequences (with the possible exception of the peptide sequence at the junction), the production of antibodies might not limit the effectiveness of repeated injections.

In conclusion, fusions between TNFSF members and collectins offer a novel means of generating large protein complexes which can provide localized stimulation at an injection site. Because of the multimeric nature of the collectin backbone, such fusion proteins may mimic the multivalent ligand surface presented by the membrane forms of TNFSF members to TNFRSF-bearing responding cells. Moreover, by limiting systemic toxicity while maintaining localized efficacy, such fusion proteins may have a role as vaccine adjuvants against infectious agents and tumors.

Table I

Ligands of the TNF Superfamily*

New Ligand Symbol	Other Names	Genbank ID
LTA	Lymphotoxin-, TNF-β, TNFSF1	X01393
TNF	TNF-α, TNFSF2	X02910
LTB	Lymphotoxin-, TNFSF3	L11016
TNFSF4	OX-40L	D90224
TNFSF5	CD40L, CD154. Gp39, T-BAM	X67878
TNFSF6	FasL	U11821
TNFSF7	CD27L, CD70	L08096
TNFSF8	CD30L	L09753
TNFSF9	4-1BBL	U03398
TNFSF10	TRAIL, Apo-2L	U37518
TNFSF11	RANKL, TRANCE, OPGL, ODF	AF013171
TNFSF12	TWEAK, Apo-3L	AF030099
TNFSF13	APRIL	NM_003808
TNFSF13B	BAFF, THANK, BLYS	AF136293
TNFSF14	LIGHT, HVEM-L	AF036581
TNFSF15	VEGI	AF039390
TNFSF16	unidentified	
TNFSF17	unidentified	
TNFSF18	AITRL, GITRL	AF125303

*(as of Nov. 1, 1999)

Known members of ligands in the TNF superfamily, taken from the Human Gene Nomenclature Committee ~~at http://www.gene.ucl.ac.uk/users/hester/tnftop.htm~~.

Table II

The Collectin Superfamily

C1q	Pulmonary surfactant protein D
Mannose-binding protein, MBL1	conglutinin
Mannose-binding protein, MBL2	collectin-43
Pulmonary surfactant protein A	CL-L1
	ACRP30
	Hib27

All collectins are formed as multimers of trimeric subunits, each containing a collagenous domain. The C-terminus of each collectin contains a CRD which binds carbohydrates and other ligands. Because of the tight similarities between the known CRD structures and the extracellular domains of TNFSF members, it is likely that the CRD of any collectin could be replaced with the extracellular domain of any TNFSF member in a structurally compatible manner.

While the present invention has now been described in terms of certain preferred embodiments, and exemplified with respect thereto, one skilled in the art will readily appreciate that various modifications, changes, omissions and substitutions may be made without departing from the spirit thereof. It is intended, therefore, that the present invention be limited solely by the scope of the following claims.